Clinica Chimica Acta 498 (2019) 161–166

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Screening and mutation analysis of hyperphenylalaninemia in newborns from Xiamen, China

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ARTICLE INFO

Keywords:
Hyperphenylalaninemia
Newborn screening
Tandem mass spectrometry
Mutation spectrum
Xiamen

ABSTRACT

In this study, we evaluated the incidence and genetic characteristics of hyperphenylalaninemia (HPA) in Xiamen, China. We analyzed the newborn screening data of HPA, obtained using a fluorometric method and tandem mass spectrometry (MS/MS), from 2013 to 2017. The suspected positive samples were further diagnosed using MassArray technology, multiplex ligation-dependent probe amplification (MLPA), and Sanger sequencing. A total of 418,831 newborns were screened, of whom 19 were diagnosed as HPA patients, with an incidence of 1:22,044. Of these HPA patients, 15 tested positive for phenylketonuria (PKU, 1:27922), and 4 tested positive for tetrahydrobiopterin deficiency (BH4D, 1:104,708). A total of 17 mutations were identified among 38 alleles in the 19 patients, with a detection rate of 94.74%, including 13 *PAH* and 4 *PTS* mutations. Among these, the c.721C > T, c.728G > A, c.1197A > T, c.611A > G and c.331C > T mutations, and the c.259C > T and c.155A > G mutations were the most prevalent *PAH* and *PTS* mutations in Xiamen, respectively. Therefore, this study systematically demonstrated the incidence and mutation spectrum of HPA in Xiamen. This information would contribute to genetic counseling, prenatal diagnosis, and management of HPA patients. Moreover, combining MS/MS technology with molecular genetic diagnosis is an effective strategy for future newborn HPA screening in Xiamen.

1. Introduction

Hyperphenylalaninemia (HPA) is the most common inborn error of amino acid metabolism, with an average incidence of 1:11,614 live births in mainland China [1,2]. It is characterized by persistent elevation of blood phenylalanine (Phe) levels (> 120 μmol/L), resulting in impaired cognitive development and neurophysiological function. HPA is clinically classified into two types: phenylketonuria (PKU, MIM # 261600) and tetrahydrobiopterin deficiency (BH4D). Approximately 98% of HPA patients have PKU. PKU is characterized by the deficiency of phenylalanine hydroxylase (PAH), an enzyme that facilitates the conversion of phenylalanine to other necessary compounds in the body [3,4]. Meanwhile, the remaining 2% of HPA patients present with BH4 deficiency (BH4D). The biosynthesis and regeneration of BH4 requires five essential enzymes: GTP cyclohydrolase I (GTPCH I, MIM # 233910), 6-pyruvoyl-tetra-hydropterin synthase (PTPS, MIM # 261640), sepiapterin reductase (SR, MIM # 612716), pterin-4α-carbinolamine dehydratase (PCD, MIM # 264070), and dihydropteridine reductase (DHPR, MIM # 261630), encoded by *GCH1*, *PTS*, *SPR*, *PCBD1*, and *QDPR*, respectively [5,6]. Patients with BH4D usually present with severe phenotypic abnormalities, as well as a poor prognosis if accurate treatment is not administered timeously [1].

Abbreviations: HPA, hyperphenylalaninemia; PKU, phenylketonuria; Phe, phenylalanine; PAH, phenylalanine hydroxylase; PAHD, phenylalanine hydroxylase deficiency; BH4D, tetrahydrobiopterin deficiency; GTPCH I, GTP cyclohydrolase I; PTPS, 6-pyruvoyl-tetra-hydropterin synthase; SR, sepiapterin reductase; PCD, pterin-4α-carbinolamine dehydratase; DHPR, dihydropteridine reductase; MHP, mild hyperphenylalaninemia; NGS, next generation sequencing; MS/MS, tandem mass spectrometry; MLPA, multiplex ligation-dependent probe amplification; DBS, dried blood spot

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https://doi.org/10.1016/j.cca.2019.08.021
Received 3 July 2019; Received in revised form 13 August 2019; Accepted 21 August 2019
Available online 22 August 2019
0009-8981/

Therefore, early differential diagnosis of HPA patients (whether PKU or BH4D) is essential to ensure that appropriate treatment is provided.

Newborn screening programs offer a simple and cost-effective method for the early diagnosis of HPA. All infants should undergo newborn screening for blood Phe concentration 72 h after birth, using a fluorometric method and/or tandem mass spectrometry (MS/MS). The commonly adopted blood Phe cut-off value is 120 μmol/L (with a Phe/Tyr ratio > 2.0). Blood Phe levels above 120 μmol/L indicate HPA. According to the blood Phe levels of untreated patients, HPA can be classified as follows: mild HPA (120–360 μmol/L), mild PKU (360–600 μmol/L), moderate PKU (600–1200 μmol/L), and classic PKU (> 1200 μmol/L) [7].

Currently, various molecular methods have been applied to the genetic testing of HPA, including conventional Sanger sequencing, microarray technology, multiplex ligation-dependent probe amplification (MLPA), and next generation sequencing (NGS) [8–12]. Implementation of these molecular methods in clinical settings can help physicians promptly identify patients' exact genotypes, and subsequently determine the appropriate and precise treatment. The frequency and distribution of HPA mutations vary significantly across different regions and populations [10,11,13–15]. A comprehensive understanding of the incidence and mutation spectrum of HPA can facilitate accurate diagnosis, individualized treatments, and genetic counseling for patients. In this study, we sought to demonstrate the screening and diagnostic principle of HPA in Xiamen Newborn Screening Center, as well as determine the incidence and mutation spectrum of HPA in 418,831 newborns in Xiamen from 2013 to 2017.

2. Materials and methods

2.1. Sample collection

In this descriptive-retrospective study, a total of 418,831 newborns were screened from 2013 to 2017 in Xiamen region. Neonatal heel prick blood specimens were collected between 3 and 7 days of life, with more than 6 intakes, by the well-trained nurses, except for the premature or sick newborns (between the first and second of life). The blood specimens were spotted and dried on Whatman 903 filter paper (Guthrie card). Then the dried blood spot (DBS) samples were delivered to the Newborn Screening Center of Xiamen Maternal and Child Health hospital every third day in the manner of cold-chain transportation. The research protocol was approved by the Ethics Committee of Xiamen Maternal and Child Health hospital. And the informed consents were obtained from all parents or their guardians.

2.2. Methods of newborn screening

The newborn screening of HPA was based on the detection of the plasma Phe concentration. The valid DBS samples were punch approximately 3.2 mm (1/8 in.) in diameter, then the Phe concentration in DBS samples were measured quantitatively by fluorometric method and tandem mass spectrometry (MS/MS) in the study.

2.2.1. Fluorometric method

A fluorometric method was used to quantitatively measure the phenylalanine in DBS samples using Neonatal Phenylalanine kit (PerkinElmer, USA). The assay operation was performed according to the manufacturer's protocol. Firstly, calibrators, high control (HC), low control (LC) and DBS samples simultaneously incubated with extraction solution for 30–60 min at room temperature and then 40 μL of deionized water were added into each well. Then 25 μL of the contents were transferred to the corresponding well of the white microplate, and 50 μL of reconstituted PKU reagent were added to each well with gently mixing the contents. After the incubation for 120–140 min at 37 °C, 200 μL copper reagent were added into each well and incubated for 30–90 min at room temperature without shaking. The fluorescence in each well is then measured via DEFIA-1420 Semi-auto time-resolved fluoroimmunoassay analyzer (Wallac, Finland). Subjects with Phe concentration > 2.0 mg/dL were contacted and collected another DBS specimen for re-evaluation.

2.2.2. Tandem mass spectrometry method

In May 2016, our screening center introduced the tandem mass spectrometry (MS/MS) system to measure the amino acids, succinylacetone, free carnitine, and acylcarnitines in DBS samples. The NeoBase Non-derivatized MSMS kit (PerkinElmer, USA) is capable to simultaneously measure 11 amino acids, and 31 carnitines and succinylacetone in DBS samples. The assay operation was performed according to the manufacturer's protocol. First, blank control (BC), high control (HC), low control (LC) and DBS samples simultaneously incubated with daily working solution (Amino Acids Internal Standards 100 μL, Acylcarnitines Internal Standards 100 μL, Succinylacetone Assay Solution 275 μL in 10.525 mL Extraction Solution). After the incubation for 45 min at 45 °C with a shaking speed of 650–750 rpm, 75 μL contents of each well were transfer into the V-bottomed, heat-resistant microplate and covered with aluminum foil to minimize evaporation of the solution. If measuring the succinylacetone, it should be reacted in stationary condition for at least 2 h to ensure complete derivatization of extracted succinylacetone. Lastly, the concentrations of Phe and Tyr in DBS samples were measured by an ACQUITY TQD mass spectrometer (Waters, Milford, MA, USA) and then the data were analyzed through the MassLynx 4.1 version software. Subjects with Phe level > 120.0 μmol/L and the Phe/Tyr ratio > 2.0 were contacted and collected another DBS specimen for re-evaluation.

2.3. Classification of HPA

In order to make a differential diagnosis between PAH deficiency (PAHD) and BH4D, BH4 loading test, along with the pterine profile analysis in urine samples (biopterin, neopterin, and percentage of biopterin), the evaluation of dihydropteridine reductase (DHPR) activity in blood samples should be performed simultaneously by Xin Hua Hospital affiliated Shanghai Jiao Tong University Medical School.

2.4. Genotyping analysis

The MassArray platform was used to test HPA-associated genes in this study, which was conducted by Genuine Diagnostics Company Limited (Hangzhou, Zhejiang Province, China). It is based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technology. In brief, DBS or peripheral whole blood of suspect-positive individuals were collected and send to the laboratory of Genuine Diagnostics Company Limited. The genomic DNA was extracted and quantified according to the manufacturer's protocol, and then were taken for the MassArray analysis. The screening panel can analyze 171 mutations of three HPA-associated genes, including 130 mutations in *PAH* gene, 38 mutations in *PTS* gene, and 3 mutations in *QDPR* gene. Mutations targeted are amplified by a multiplex PCR assay, and then unincorporated dNTPs were digested by the addition of shrimp alkaline phosphatase (SAP) to the PCR reaction product. Single base extension was performed by using extension primers and mass-modified dideoxynucleotide terminator. Subsequently, the products were directly spotted in 384 holes on genotyping chip, and the mass of the extended primer was analyzed with MALDI-TOF mass spectrometry. The assay results were then automatically read by the MassARRAY real-time software system and the genotypes were obtained from the MassARRAY Typer software. All the mutations identified by MassArray were further validated by Sanger sequencing of the patients.

When one or no *PAH* mutation(s) was identified in PKU patients, multiplex ligation-dependent probe amplification (MLPA) analysis were subsequently used to analyze the *PAH* copy-number variants. The SALSA P055 PAH MLPA kit (MRC-Holland, Amsterdam, Netherlands)

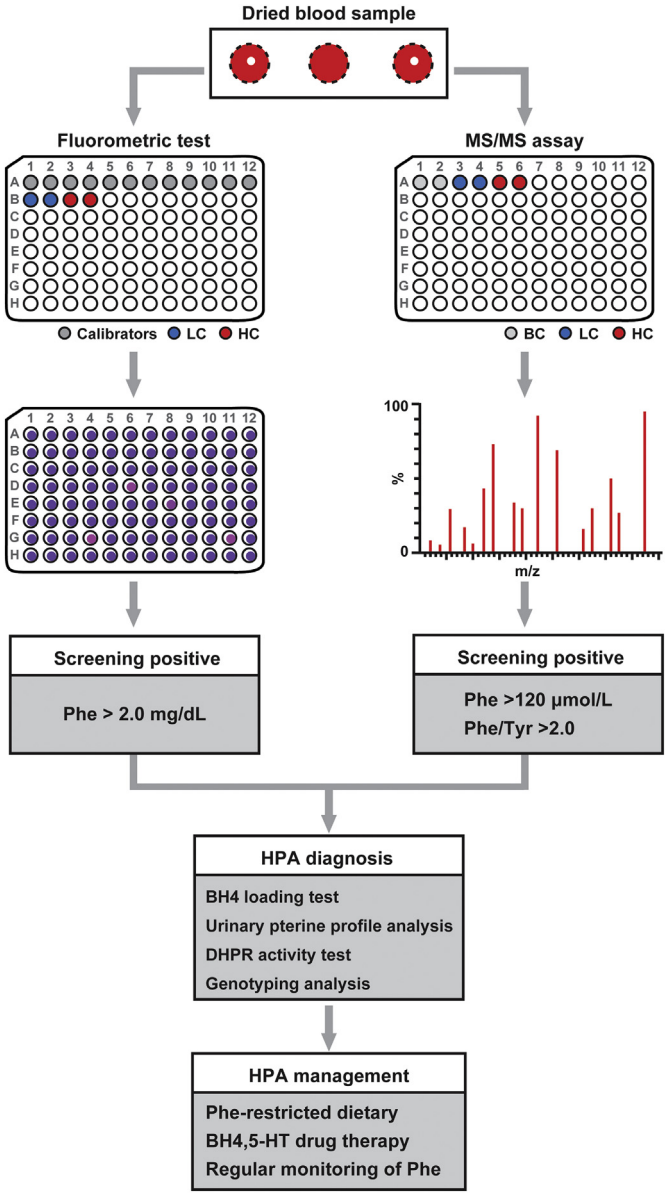

Fig. 1. Workflow of newborn screening for hyperphenylalaninemia. The newborn screening of hyperphenylalaninemia was based on detection of the plasma Phe level, which is measured quantitatively by a fluorometric method and/or MS/MS assay. Thereafter, screening of positive samples is performed using a BH4 loading test, urinary pterine profile analysis, a DHPR activity test, and genotyping analysis to determine differential diagnosis and treatment methods for HPA patients. LC and HC were high- and low-level controls of the fluorometric method and MS/MS assay, respectively.

was used to analyze the deletions or duplications in *PAH* gene according to the manufacturer's instruction. The amplification products were electrophoresed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and raw data were analyzed with the Coffalyzer software (MRC-Holland). In brief, the fluorescent signals were compared to normal controls, resulting in a ratio of 0.40–0.65 for deletions and 1.30–1.65 for duplications.

3. Results

3.1. Newborn screening for HPA

During this five-year-long descriptive-retrospective study, the HPA screening platform of Xiamen region mainly consisted of newborn screening for Phe levels, diagnosis and management of HPA, and regular monitoring of Phe levels (Fig. 1). From January 2013 to December 2017, a total of 423,545 live infants were born in the Xiamen region. Of them, 418,831 (98.89%) and 52,815 (30.21%) were screened for the phenylalanine levels using a fluorometric method and MS/MS assay, respectively (Table 1). In the first screening, 27 newborns were screened for suspected positive results with Phe concentration > 2.0 mg/dL by the fluorometric assay, of whom 25 were successfully recalled for diagnosis (92.6%), and 19 were finally confirmed to have HPA (positive predictive value = 76.0%). Additionally, 9 newborns were screened for HPA-positive results by the MS/MS assay; these newborns were all successfully recalled (100.0%) and finally diagnosed with HPA. Thus, the average HPA incidence in the Xiamen region during the five-year period (2013–2017) was approximately 0.05‰ (1:22,044).

3.2. Genotyping analysis of HPA

Based on biochemical analysis, molecular diagnosis, and clinical symptoms, 19 newborns were diagnosed with HPA, specifically 15 with PAHD and 4 with PTPS deficiency (Table 2). Among the 15 PAHD patients, the genotyping test identified 13 patients with two mutations in two alleles of *PAH*, and 2 patients with only one mutation in one allele. Thus, the mutation detection rate among the 30 *PAH* alleles was 93.33% (28/30). Specifically, a deletion spanning the 5′-UTR and exon 1 was detected in one patient by MLPA and confirmed by Sanger sequencing (Fig. 2). In addition, the 4 PTPS-deficient patients carried two mutations in two alleles of *PTS*. All mutations were finally confirmed by Sanger sequencing.

4. Discussion

Newborn screening for defects in phenylalanine metabolism is an efficient method for early detection and prompt treatment of HPA, which can greatly improve the survival and well-being of affected individuals. The introduction of MS/MS technology is one of the most significant advances in screening neonates for HPA. This technology, characterized by high throughput, specificity, and sensitivity, has been

Table 1
Newborn screening coverage, screened positives, confirmed positives, and incidence of hyperphenylalaninemia in Xiamen (2013–2017).

Year	Number of newborns (n)	Fluorometric method				MS/MS method				Incidence (‰)
		Number screening (n)	Coverage (%)	Screened positives (n)	Confirmed positives (n)	Number screening (n)	Coverage (%)	Screened positives (n)	Confirmed positives (n)	
2013	79,913	78,250	97.91	6	4	–	–	–	–	0.05
2014	84,952	84,185	99.10	4	3	–	–	–	–	0.04
2015	83,849	82,979	98.96	5	3	–	–	–	–	0.04
2016	86,985	86,204	99.10	7	4	16,974	19.51	2	2	0.05
2017	87,846	87,213	99.28	9	5	35,841	40.80	5	5	0.06
Total	423,545	418,831	98.89	27	19	52,815	30.21	9	9	0.05

Table 2
Genotypes and phenotypes of 19 HPA patients from Xiamen, China.

Case	Gender	Feeding (> 6 intakes)[a]	Phe (mg/dL)[b]	MS/MS		Genotype	Phenotype[c]
				Phe (μmol/L) (21 − 101)	Phe/Tyr (0.15–1.20)		
P01	M	Y	13.5	517.51	6.34	*PTS*: c.259C > T/c.259C > T	Mild
P02	M	Y	10.2	762.04	9.66	*PAH*: c.721C > T/N	Moderate
P03	M	Y	8.3	538.31	8.68	*PAH*: c.721C > T/c.728G > A	Mild
P04	F	Y	20.8	1460.20	25.18	*PAH*: c.331C > T/c.1197A > T	Classic
P05	M	Y	8.3	324.56	5.87	*PTS*: c.155A > G/c.286G > A	MHP
P06	M	Y	16.4	893.60	9.47	*PAH*: c.611A > G/c.1197A > T	Moderate
P07	F	Y	9.2	614.64	11.56	*PAH*: c.721C > T/c.1049C > A	Moderate
P08	M	Y	17.6	927.43	17.44	*PAH*: c.728G > A/E1 del3.7Kb	Moderate
P09	M	Y	12.3	636.98	6.33	*PAH*: c.913-7A > G/N	Moderate
P10	M	Y	21.4	1502.04	24.2	*PAH*: c.728G > A/c.1197A > T	Classic
P11	M	Y	4.8	196.61	3.22	*PAH*: c.721C > T/c.721C > T	MHP
P12	F	Y	2.8	152.50	2.13	*PAH*: c.320A > G/c.722G > A	MHP
P13	M	Y	7.4	433.14	5.78	*PAH*: c.721C > T/c.1071C > A	Mild
P14	M	Y	6.3	352.17	4.97	*PAH*: c.721C > T/c.728G > A	MHP
P15	M	Y	2.2	142.51	2.20	*PAH*: c.331C > T/c.1174T > A	MHP
P16	F	Y	9.2	531.84	6.13	*PTS*: c.155A > G/c.259C > T	Mild
P17	M	Y	3.1	239.80	3.61	*PTS*: c.84-291A > T/c.259C > T	MHP
P18	M	Y	22.4	1252.29	23.7	*PAH*: c.611A > G/ c.611A > G	Classic
P19	M	Y	12.6	923.61	6.03	*PAH*: c.721C > T/c.1238G > C	Moderate

[a] Feeding status of the patients at the time of blood collection in the first screening, Y stands for Yes.
[b] Phe at birth quantified by the fluorometric method, 1 mg/dL = 60.5 μmol/L.
[c] Phenotype was classified according to Phe concentrations determined by the MS/MS assay. Classic, classic PKU (Phe, > 1200 μmol/L); moderate, moderate PKU (Phe, 600–1200 μmol/L); mild, mild PKU (Phe, 360–600 μmol/L); MHP, mild hyperphenylalaninemia (Phe, 120–360 μmol/L).

widely applied for the detection of inherited metabolic disorders (IMDs) [12]. MS/MS technology surpasses the fluorometric method, as it enables simultaneous detection of Phe levels, Tyr levels, and the Phe/Tyr ratio, significantly reducing the false-positive rate in HPA screening. Moreover, the MS/MS technology may detect more than 30 IMDs (including amino acid, fatty acid, and organic acid disorders) in a single test at a relatively low cost, comfortably satisfying the needs of newborn screening programs.

HPA incidence varies greatly among different countries and populations. It was reported that HPA has an incidence of 1:19,000–1:13,500 in newborns in the United States [16], 1:10,000 in Europe [17], 1:8690 in Brazil [18], 1:70,000 in Japan [19], and 1:36,000–1:327,000 in Thailand [20]. In mainland China, the overall incidence of PKU is approximately 1:12,000 [2,21]. In Taiwan, the average incidence of HPA is 1:37,549, whereas that of PKU is 1:55,057 [22]. In this retrospective study, the overall incidence of HPA in Xiamen was found to be roughly 1 in 22,044 newborns; the incidence of PKU was 1:27,922, which is comparable to 1:22,045 observed in Zhejiang province [11]. Our results agree with previous findings, indicating that PKU incidence in Northern China is significantly higher than that in Southern China [10,11]. In our

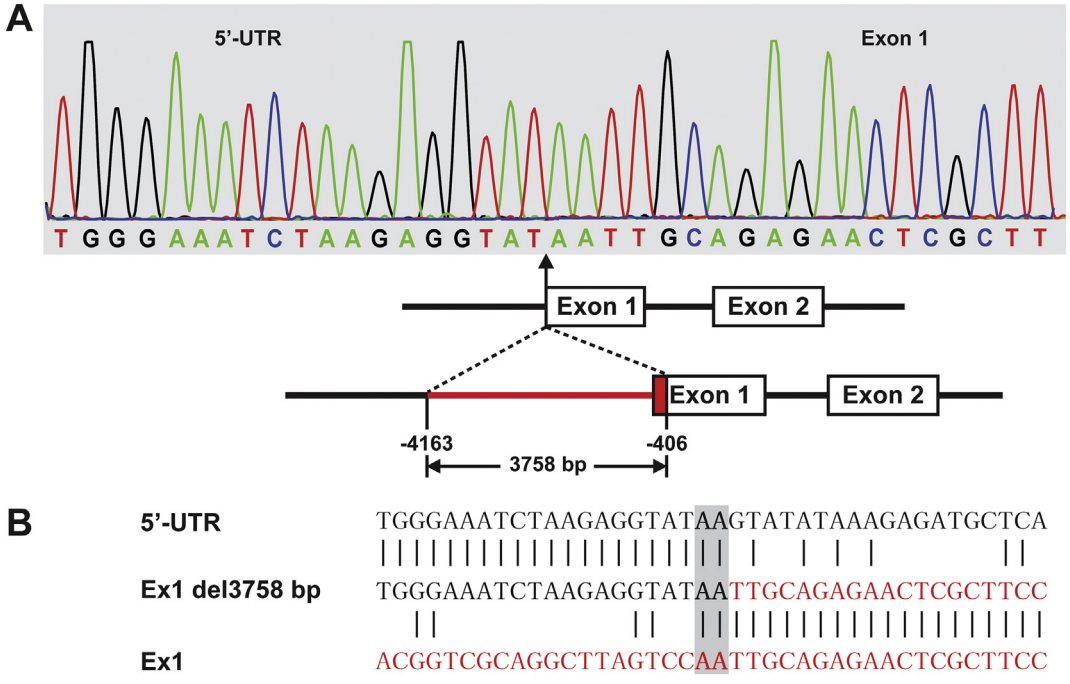

Fig. 2. Breakpoint analysis of exon 1 deletion (EX1 del 3758 bp) in sample P08. Sanger sequencing analysis (**A**) and multiple sequence alignment (**B**) revealed that this deletion spanning the 5′-UTR and exon 1 of *PAH* is identical to the deletion reported by Yan et al. (Analysis of large deletion of phenylalanine hydroxylase gene in Chinese patients with phenylketonuria. *Zhonghua Yi Xue Za Zhi*, (2016) 96.14:1097–1102).

study, 4 HPA patients were diagnosed with PTPS deficiency, accounting for 21.05% of the HPA patients. The incidence of BH4D in Xiamen was found to be 1:104,708, which is slightly higher than that in Taiwan (1:115,010) [23]. In mainland China, the majority of BH4 deficiency (240/250, 96.0%) is caused by PTPS dysfunction [24]. These findings demonstrate the increasing frequency of BH4D in China, specifically Southern China, suggesting the need for rigorous BH4D testing and diagnosis.

Mutations were detected on 36 of the 38 alleles in a cohort of 19 HPA patients, with a detection rate of 94.74% (Table 2). In total, 13 mutations were identified in *PAH*, whereas 4 were identified in *PTS*. In the 15 PKU patients, c.721C > T (p.R241C, 26.67%), followed by c.728G > A (p.R243Q, 13.33%), c.1197A > T (p.V399V, 10.00%), c.611A > G (p.EX6-96A > G, 10.00%), and c.331C > T (p.R111*, 6.67%) were the most prevalent *PAH* mutations, accounting for 71.43% of all identified mutant alleles. These results are consistent with those of previous studies; however, the frequency of the mutations varied across different regions [8,14,15,25]. In this study, c.721C > T (p.R241C, 26.67%) was found to be the most frequent *PAH* mutation in the Xiamen region, which was consistent with results obtained for Southern China and Taiwan [13,22]. Notably, c.1223G > A (p.R408Q) is a common mutation in the population of Taiwan [13], whereas it was not detected in the Xiamen population. In the 4 PTPS-deficient patients, c.259C > T (p.P87S), c.155A > G (p.N52S), c.84-291A > T (p.Y27Rfs*8), and c.286G > A (p.D96N) in *PTS* accounted for 50.00%, 25.00%, 12.50%, and 12.50% of the mutations, respectively. The distribution of *PTS* mutations observed in this study is comparable to that in previous studies on East Asian populations [26]. One *PAH* mutation was detected in 3 HPA patients, using MassArray analysis. Thereafter, MLPA was used to determine whether large deletion mutations occurred in the *PAH* gene of the 3 patients. A large deletion mutation (EX1 del 3758 bp) spanning the 5′-UTR and exon 1 of *PAH* was detected in patient P08 (further confirmed by Sanger sequencing), whereas no mutations were detected in the other 2 patients. It is probable that the mutation was out of the detection range of MassArray and MLPA, such as in the promoter region, 5′ and 3′ noncoding regions, intronic sequences, noncoding RNA-binding sites, or other unknown large deletions. Comprehensive molecular genetic diagnosis of HPA can reveal structural and functional changes in causative genes, as well as genotype-phenotype correlations. Moreover, this method of testing also allows prenatal diagnosis, and provides guidance for genetic counseling and subsequent treatment. Altogether, these advantages facilitate proper treatment to ensure normal physical and neurological development in HPA patients.

In conclusion, we demonstrated the importance of newborn screening and diagnostic principles of HPA by investigating the overall incidence and mutation spectrum of HPA in Xiamen. The data indicate that BH4D (specifically PTPS deficiency) is more prevalent in Xiamen, providing evidence that differential diagnosis of BH4D should be encouraged by physicians. The findings presented in this study highlight the importance of genetic counseling, prenatal diagnosis, and management of HPA patients in this area. More importantly, a detection model combining tandem mass spectrometry technology with molecular genetic diagnosis, as well as the cold-chain transportation of DBS samples, is of great necessity for future newborn HPA screening in the Xiamen region.

Funding

This work was supported by the Foundation for Young Scholars of Fujian Provincial Department of Health Office (Grant No. 2017–2-107), National Natural Science Foundation of China (Grant No. 81572084), Xiamen Science and Technology Major Project (Grant No. 3502Z20171006) and Xiamen Major Disease Research Project (Grant No. 3502Z20149030).

Declaration of Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

We thank the patients and their families for their contributions in this study. This work was financially supported by the Foundation for Young Scholars of Fujian Provincial Department of Health Office (Grant No. 2017-2-107), National Natural Science Foundation of China (Grant No. 81572084), Xiamen Science and Technology Major Project (Grant No. 3502Z20171006) and Xiamen Major Disease Research Project (Grant No. 3502Z20149030).

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